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Serotonin Transporter Knockout Mice and Maternal Stress: A Potential Animal Model of Autism

by

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Abstract

This study examined whether an interaction between the serotonin transporter genotype and maternal stress results in a mouse with characteristics of autism. Serotonin transporter knockout heterozygous dams and wild-type dams were stressed during different gestational periods. Their offspring then underwent a series of behavioral tasks to test sociability, anxiety levels, and level of cerebellar functioning. We predicted that mice that experienced prenatal stress and had a deficit in the serotonin transporter would produce the anxiety, cerebellar and social interaction deficiencies seen in autism. In contrast, we hypothesized that without the presence of prenatal stress, there is no difference in the baselines of the control groups and the serotonin transporter knockout mice. Our results did not support our hypothesis, suggesting that the serotonin transporter knockout mice and prenatal stress paradigm is not a valid animal model of autism. However, we did find effects on sociability, anxiety, and locomotor functioning, indicating an interaction of serotonin transporter knockout mice and maternal stress.

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Autism spectrum disorder (ASD) is a developmental disorder that is classified by impairments in social interactions and communication, as well as repetitive and restrictive behaviors (Cook, 1997; Crawley, 2004; DiCicco-Bloom, 2006; Tuchman, 2003). The etiology of ASD is largely unknown, but several factors have been suggested. By creating an animal model of autism, these factors can further be investigated.

One suggested factor contributing to the development of autism is the serotonin transporter (5-HTT). The function of 5-HTT is to uptake extracellular serotonin (5-HT) and transport 5-HT back into the cell (Murphy, 2004). Polymorphisms of the 5-HTT gene can alter 5-HTT's functioning, including uptake, binding ability, and expression (Lesch, 1996; Murphy, 2004). An insertion/deletion within the promoter region of the 5-HTT gene results in either the long or short variant polymorphism, respectively (Lesch, 1996; Murphy, 2004). It has been discovered that possessing at least one allele of the short variant of the 5-HTT gene, SLC6A4, is associated with numerous clinical conditions, including depression and anxiety (Capsi, 2003; Lesch, 1996). Harari et al (2002) discovered that carriers of the short allele show greater amygdala activation to fearful stimuli. This suggests the possibility that carriers of the short allele have greater reactivity to stressful stimuli.

Similar to the human literature, 5-HTT knockout (KO) mice exhibit anxiety-like behaviors (Holmes, 2003). These 5-HTT KO mice also display increased levels of extracellular 5-HT, decreased intracellular concentrations of 5-HT, and reduced transporter binding availability and uptake activity in comparison to wild-type and

heterozygous 5-HTT mice (Bengel, 1998; Kim, 2005; Montanez, 2003). While the heterozygous 5-HTT KO mice have similar 5-HT levels as wild-type mice, their uptake activity and transporter binding availability is reduced roughly 50% in comparison to wild-type mice (Bengel, 1998; Montanez, 2003). This reduction of the heterozygote's functioning is similar to that seen in human studies investigating the short allele of the 5-HTT gene (Lesch, 1996). Therefore, in order to best model the human 5-HTT function, heterozygous 5HTT KO mice should be used.

Another suggested factor contributing to the development of ASD is prenatal stress (Beversdorf, 2005; Kinney, 2007). In a study conducted by Beversdorf et al. (2005), it was discovered that mothers of children with autism had a significant increase in stress during prenatal weeks 21-32 in comparison to normally functioning children, as well as children with Down syndrome. It was also discovered that a peak at 25-28 weeks gestation was observed for the autistic group (Beversdorf, 2005). In investigating the effects that naturally occurring stressors have on prenatal development, Louisiana parishes were researched for their differing prevalence rates of autism. The prevalence rates increased in a storm intensity-dependent manner, especially when the hurricanes and/or tropical storms were experienced during the late 2nd or early 3rd trimester (Kinney, 2007). Both of these studies implicate prenatal stress as a possible risk factor in the development of ASD.

Acute stress produces increased levels of circulating glucocorticoids and 5-HT, which has the ability to alter fetal development (Gaspar, 2003; Leonard, 2006). In contrast, chronic stress has been shown to decrease 5-HT levels, which can be seen by measuring peripheral 5-HT blood platelet levels (Bianchi, 2002). Cote et al. (2007)

found that maternal peripheral 5-HT is crucial in the neuronal development of mice. Thus, stress-related changes in 5-HT levels may have the ability to significantly alter development of mice offspring.

While numerous studies have investigated the independent effects of prenatal stress and of increased circulating 5-HT levels on the etiology of autism, few have investigated the interactions between these factors. In addition, little is known about the effects caused by variation in the timing of prenatal stress on both neuronal development and behavioral implications. Therefore, the present study will conduct a 2x3 experiment, measuring genotype and maternal stress. The genotype conditions will consist of the 5-HTT KO mice and of wild-type mice that serve as a genetic control. The stress conditions are: no stress (control), early prenatal stress (gestational days 8-10), and late prenatal stress (gestational days 16-18). We hypothesize that without the presence of prenatal stress, there will be no difference in the baselines of the control groups and the 5-HTT KO heterozygous mice. In contrast, we hope to establish that the presence of prenatal stress and a deficit in 5-HTT expression, as seen in the 5-HTT KO mice, produces the anxiety, exploration, and social interaction abnormalities seen in autism.

Methods

Animals

Male homozygous 5-HTT KO mice with a C57BL/6 background were developed in Dennis Murphy's laboratory (Bengel et al, 1998), and were from Taconic Farms, Inc. (Hudson, NY), and crossed with heterozygous C57BL/6 females to obtain the

heterozygous dams used for this study. Three heterozygous 5-HTT KO heterozygous dams and three wild-type dams were used throughout the study. Each dam was crossed with wild-type male mice to produce offspring. The three dams in each group were subjected to one of three different stress factors: stress during gestational days 8-10, during gestational days 16-18, or no stress, serving as controls. Offspring of all dams were weaned on postnatal day (PD) 28. On PD 35, a general neurological exam (described below) was conducted on the offspring mice. Behavioral testing began on PD 60.

Prenatal Stress Paradigm

Wild-type and 5-HTT KO heterozygous dams were randomly assigned to one of the three different stress conditions. Animals assigned to the stress groups experienced the following stress conditions over a period of three days: novel object (marbles) overnight exposure, one hour fox urine exposure, 36 hours of constant light, 10 minute restraint stress, novel noise overnight, multiple cage changes throughout a 24 hour period, and saturated bedding (300 mL) overnight. These stressors have previously been shown to be effective based on corticosterone measurements, but do not cause changes in feeding or body weight (Mueller, 2006).

Neurological Battery Exam

The offspring were examined using a neurological battery to detect any deficits in sensory function or gross developmental abnormalities. If an offspring was determined to have any gross neurological abnormalities affecting sensorimotor behaviors, they were

excluded from further study. The neurological battery included forepaw extension upon being lowered to a surface, indicating vision deficits, freezing behavior following acoustic startle, indicating auditory defects, neuromuscular grip strength, and individual weight.

Apparatus and Procedure

Social behavior was tested using a 3-chamber social approach task described by Nadler et al (2004). The apparatus contained 3 separate chambers measuring 21cm x 43 cm. The middle chamber served as the start box, while the outer chambers were used for the experimental manipulations. Time spent in each chamber was then recorded for later analysis across three trials. Mice were first exposed to the center chamber for ten minutes to habituate to the apparatus. The first trial served to establish the presence or absence of a side bias. The second trial measured the sociability levels of the experimental mice. An inverted pencil cup with wire slotted slides, a novel object, was placed in both outside chambers. In one chamber, a stranger mouse was placed under the cup, so that the experimental mouse had the option of interacting or avoiding this stranger mouse. The cup in the other chamber was left empty (see Figure 1). The amount of time in each chamber was measured, and more time spent in the chamber of the stranger mouse was indicative of greater sociability. A novel stranger mouse was placed in the previously empty cup in the final trial, and the experimental mouse was allowed to explore the entire apparatus again. More time spent with the novel stranger was indicative of increased social novelty seeking and increased recognition of social cues.

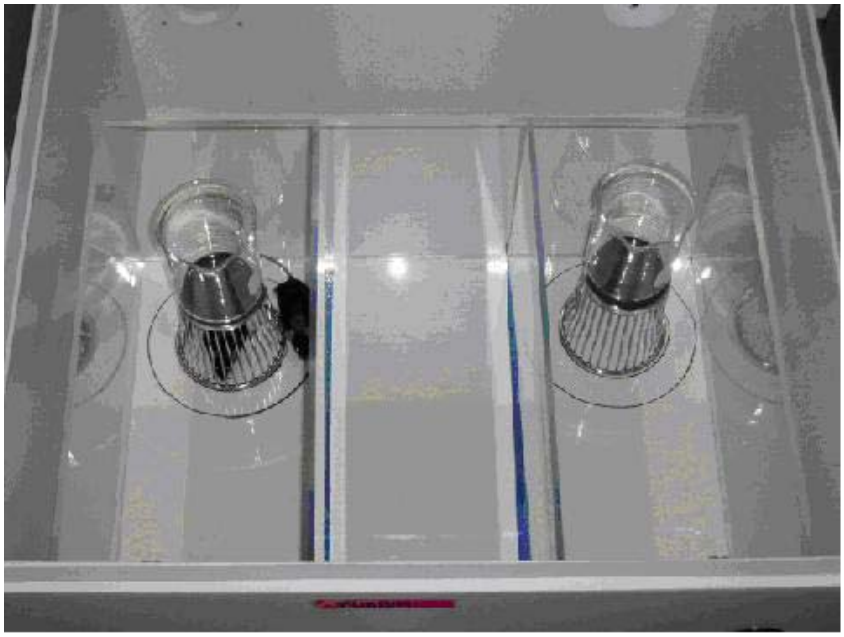

Fig. 1. 3-chamber social approach task stranger versus novel object trial

To test anxiety-like behavior, the mice were tested in an elevated-plus maze (EPM). The EPM contained two open arms (30 x 5 x 0.25 cm^3) and two closed arms (30 x 5 x 15 cm^3) that extend from a common central platform (5 x 5 cm^2). The apparatus was constructed from Plexiglas and was elevated 60 cm above the floor (see Figure 2). Each mouse was allotted 5 minutes to explore the apparatus. Time spent on each arm and entries onto the open and closed arms, as well as head dips of the side of the open arms, were recorded. Time spent in the open arms typically indicates low anxiety whereas time spent in the closed arms indicates higher anxiety. Therefore, to measure anxiety, we used a ratio of open arm time over total arm time.

Another measure testing anxiety levels measured the willingness of the mouse to explore a novel open-field environment, and also allowed for the quantification of

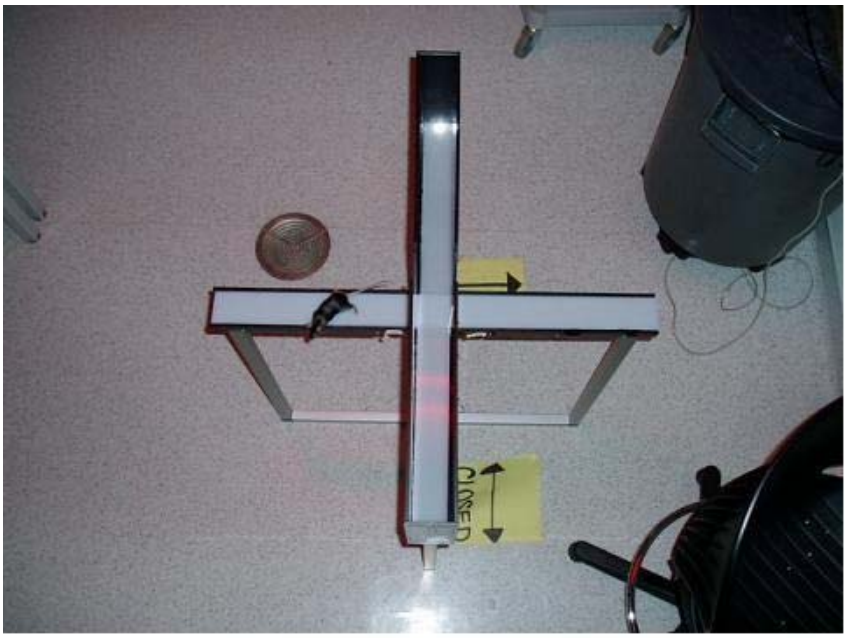

Figure 2. Elevated-Plus Maze

locomotion. The open field areas measured 50 x 50 cm. A square grid was placed underneath the floor to mark the different quadrants of the open field. Mice were given a total of 10 minutes to explore. Anxious behavior was quantified as the time spent in the corner and perimeter quadrants versus the interior quadrants of the grid.

In the final behavior task, the mice were tested using a rotarod. This task assesses the locomotor and cerebellar functioning of the mice. Prior to behavioral testing, the mice were given three test trials in order to familiarize themselves with the apparatus. Testing trials were at three different constant speeds: 16 rotations per minute (rpm), 24 rpm, and 32 rpm. The following day, mice were tested on the rotarod for up to five minutes at an accelerating speed, beginning at 4 rpm and reaching up to 40 rpm at the end of the trial. Three trials were recorded and scored. A shorter latency to fall was indicative of poor locomotor and cerebellar functioning.

Statistical Analysis

A repeated measures ANOVA was used to analyze the available data. Significance was set at $P \leq 0.05$. Bonferroni multiple comparisons tests determined post hoc significance when appropriate. Subsequent t-tests was then completed to identify any effects or interactions revealed by the ANOVA. All statistical analysis was performed using SPSS.

Results

Elevated-Plus Maze

Anxiety levels were assessed as an open arm ratio, and were calculated as the ratio of time spent on the open arms over total arm time. 5-HTT KO mice prenatally stressed late in gestation showed less anxiety as indicated by an increased open arm ratio in comparison to all other groups ($P < 0.01$). Bonferroni multiple comparisons post hoc tests showed significance for the prenatally late stressed 5-HTT KO offspring in comparison to early and control 5-HTT KO mice, as well as wild-type control offspring (see Figure 3). Subsequent t-tests revealed a trend between the late stress 5-HTT KO group, compared to the wild-type control group ($P < 0.09$).

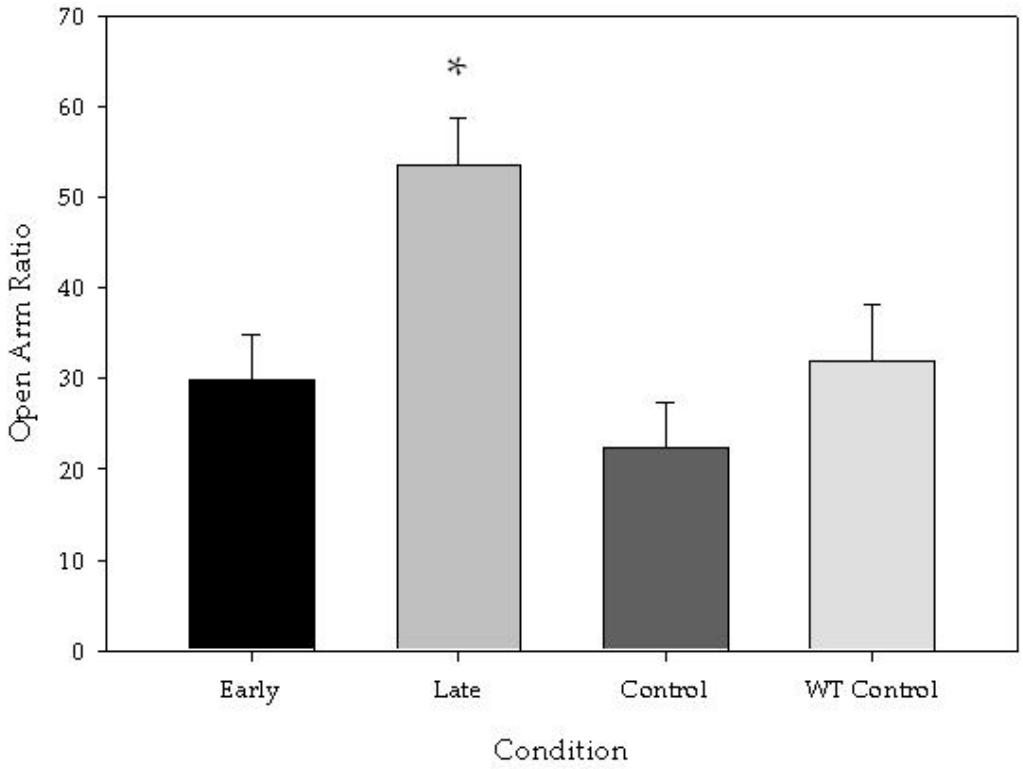

Fig. 3. *Time spent in open arm over time spent in open and closed arms as a function of stress and genotype conditions in the elevated-plus maze..*
**Significance for late stress condition relative to early stress, SERT control, and WT control conditions (P<0. 05)*

Open Field

A significant main effect of open field testing was observed, as mice spent a greater amount of time in the outer quadrants of the open field than in the inner quadrants ($P < 0.01$). A significant condition and field interaction was also observed ($P < 0.04$). This effect was further supported through subsequent t-tests, which revealed that the late stress 5-HTT KO group was significantly more anxious than the 5-HTT KO control group ($P < 0.03$). A trend was also observed for the early stress 5-HTT KO mice in comparison to the 5-HTT KO control group ($P < 0.09$) (see Figure 4).

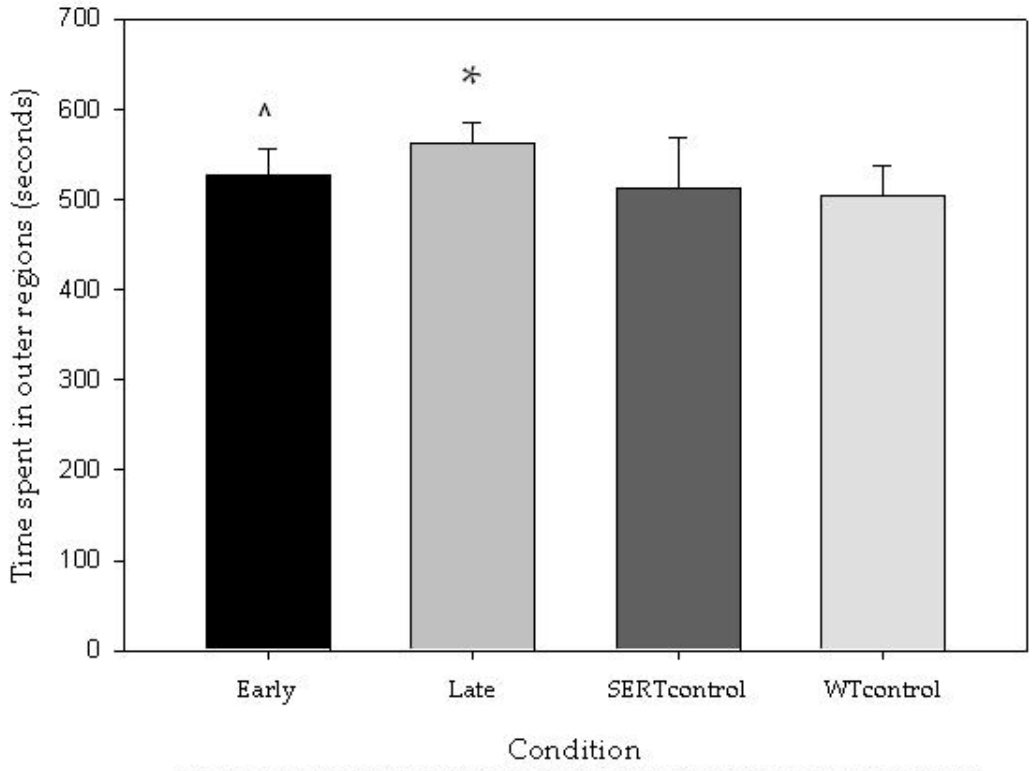

Figure 4. *Time spent in outer quadrants in the open field task as a function of genetic and stress condition.*
**The late stress 5-HTT KO mice group was significantly more anxious in comparison to the 5-HTT KO control group ($P < 0.05$).*
^The early stress 5-HTT KO mice group were more anxious in comparison to the 5-HTT control group (as seen by a trend of $P < 0.09$)

Three-chamber social approach task

Two separate repeated measures ANOVA were completed. The first ANOVA analyzed the stranger versus novel object trial, and the second ANOVA analyzed the trial during which both stranger mice were present. In the one stranger trial, a significant main effect for chamber was observed ($P < 0.01$). Pairwise comparisons revealed significance between all three chambers with respect to each other ($P < 0.01$). Mice spent most of their time in the chamber with the stranger mouse, less time in the chamber with the novel object, and the least amount of time in the center chamber. In addition, male mice spent significantly more time with the stranger mouse than with the novel object in comparison to females ($P < 0.05$) (see Figure 5).

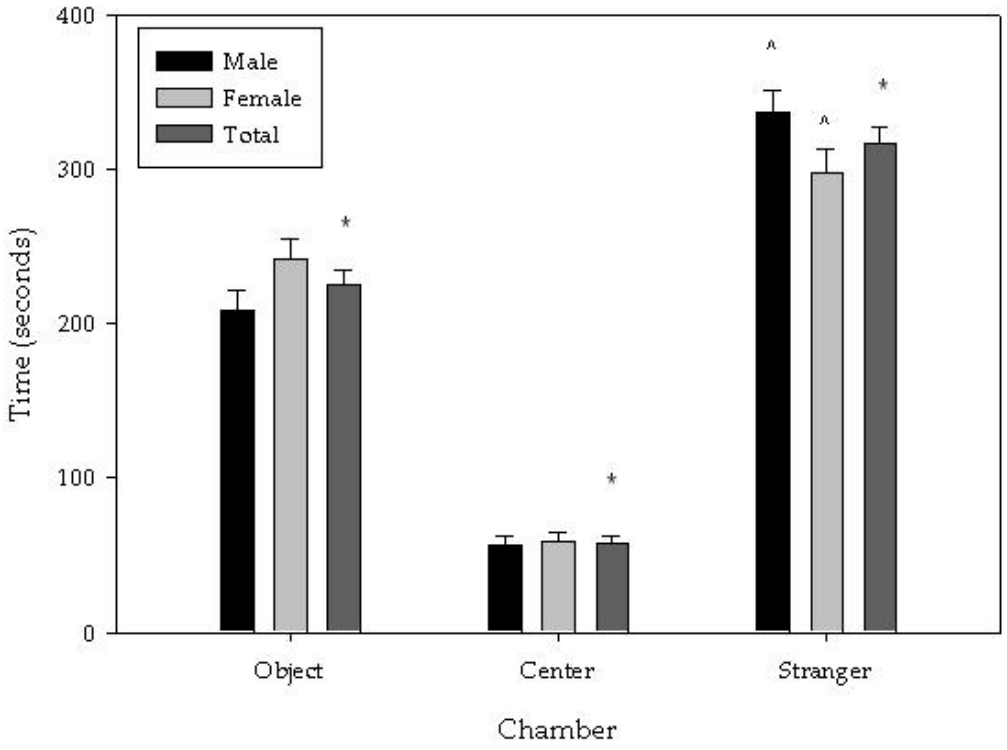

Figure 5. *Time spent in the 3 chambers as a function of sex in the 3-chamber social approach task..*
**A significant main effect of chamber was seen (P < 0.01).*
^A significant chamber by sex interaction was observed (P < 0.05).

Subsequent t-testing also showed a significance between early and late stressed 5-HTT KO offspring, between early stressed and control 5-HTT KO offspring, between early stressed 5-HTT KO and wild-type control offspring, between late stressed and control 5-HTT KO offspring, and between late stressed 5-HTT KO and wild-type offspring in respect to all chambers (see Figure 6).

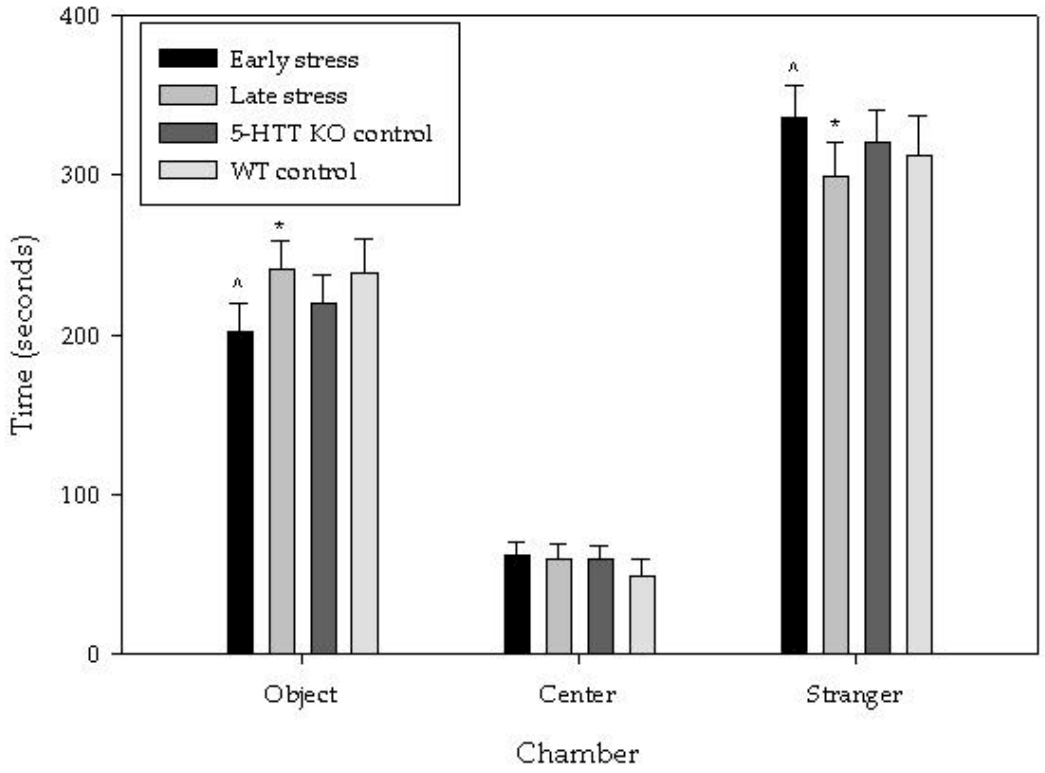

Fig. 6 *Time spent in 3-chambers as a function of genetic and stress conditions in the 3-chamber social approach task.*
** 5-HTT KO mice prenatally stressed late showed a significant difference in time in object and stranger chambers in comparison to all other stress and genotype conditions*
^ 5-HTT KO mice prenatally stressed early showed a significant difference in time in object and stranger chambers in comparison to all other stress and genotype conditions

In trials when two stranger mice present, a significant main effect of chamber was observed ($P < 0.01$). Mice spent significantly less time in the center chamber than either of the chambers containing stranger mice. In addition, males spent more time socially

interacting with a novel stranger than females, revealing a significant main effect of sex ($P < 0.02$). Bonferroni post hoc analysis revealed significance between 5-HTT KO control mice and wild-type control mice ($P \leq 0.05$). Subsequent t-tests showed a significant difference in the late stress 5-HTT KO group and the 5-HTT KO control group, compared to the time spent with the non-novel stranger mouse. Overall, trends were observed in the following: early stressed 5-HTT KO mice versus late stressed 5-HTT KO mice, and late stressed 5-HTT KO mice versus wild-type control mice (see Figure 7).

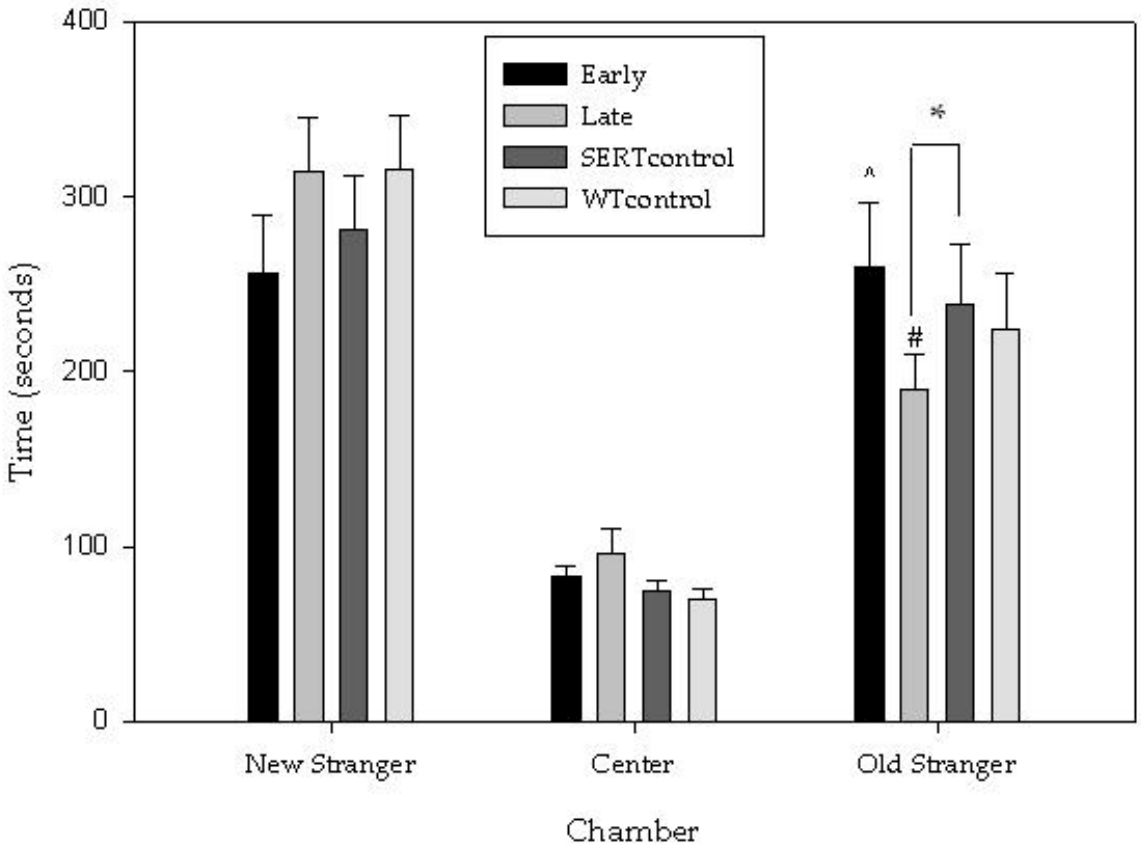

***Figure** 7. Time spent in three chambers as a function of genetic and stress conditions in the 3-chamber social approach task.*
**5-HTT KO mice prenatally stressed late spend less time with a familar stranger than 5-HTT control mice*
^5-HTT KO mice prenatally stressed early spend moretime with a familar stranger than 5-HTT late stress mice
#5-HTT KO mice prenatally stressed late spend less time with a familar stranger than WT control mice

Rotarod

Wild-type control mice data have not yet been collected for this measure. Significant main effects were observed for trial, sex, and condition ($P < 0.05$). The early stressed 5-HTT KO group performed significantly worse than the 5-HTT KO control group and a trend was observed between the early versus late stressed 5-HTT KO groups (see Figure 8). Males had a significantly faster latency to fall than females, based upon pairwise comparisons ($P < 0.01$). The first trial was also significant compared to Trials 2 and 3, as mice generally improved their performance, and had longer latencies to fall. Bonferroni post hoc tests showed a significant difference between prenatally early stressed 5-HTT KO mice and 5-HTT control mice.

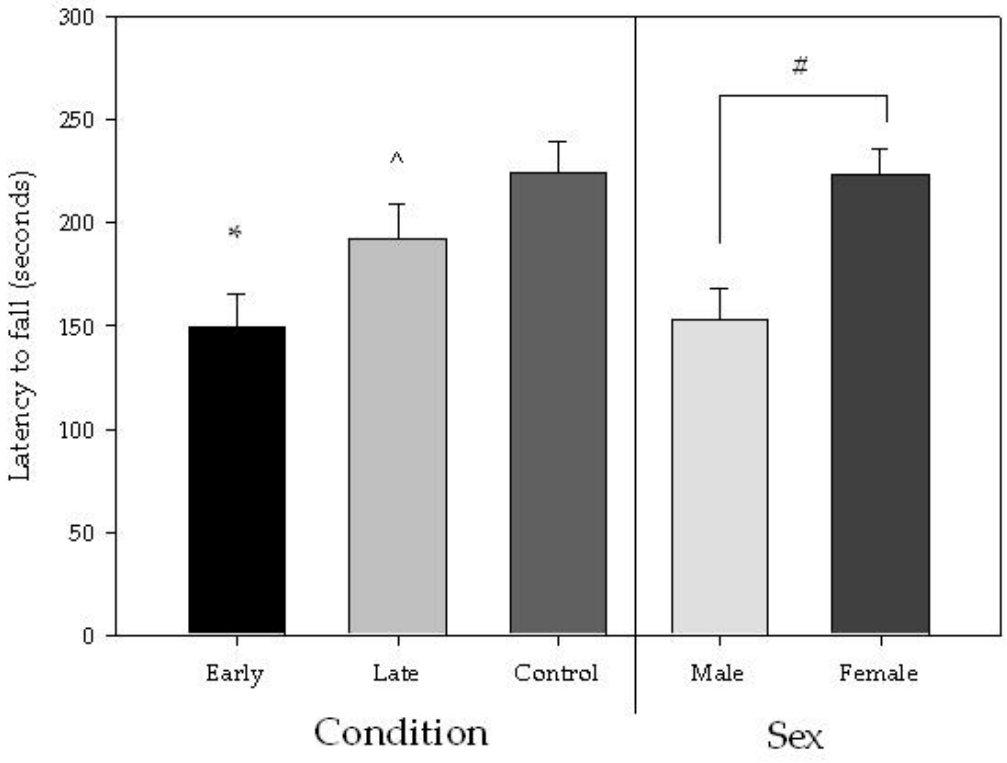

Figure 8. *Latency to fall in the rotarod task as a function of both stress condition and sex.*
**Early prenatal stress 5-HTT KO group had a significantly faster latency to fall than 5-HTT control mice.*
^Early prenatal stress 5-HTT KO group had a faster latency to fall than the 5-HTT late group ($P < 0.09$)
#Male mice performed significantly worse than female mice ($P < 0.01$).

Discussion

To create a valid animal model of a psychological disorder, behavioral results must be stereotypical of the disorder and easily reproducible. It appears that our suggested stress by genotype paradigm is an incomplete animal model of the symptoms of autism for those reasons.

The two tasks used to measure levels of anxiety, the EPM and open field task, produced contradictory results. Prenatally late stressed 5-HTT KO mice displayed behavior indicative of decreased levels of anxiety, compared to both wild-type and 5-HTT KO control groups in the EPM task. In contrast, the prenatally late stressed 5-HTT KO mice spent less time in the inner quadrants of the open field task than control groups, indicative of increased levels of anxiety during the open field task. The same results have been demonstrated in a study by Kwon et al. (2006). Kwon suggests that this may reflect different controlling neural networks for these two types of anxiety-like behaviors.

The three-chamber social approach task is a behavioral measure meant to assess levels of social interaction in mice. More time spent in the chamber with a stranger mouse in comparison to a novel object is indicative of greater sociability. In the trial in which two stranger mice are present, total time spent with the novel stranger can be interpreted as increased social novelty seeking and increased recognition of social cues. Through analysis of both types of trials, the prenatally late stressed 5-HTT KO mice had increased levels of sociability, social novelty seeking and recognition of social cues in comparison to all other conditions. This contradicts our hypothesis, as we expected late stressed 5-HTT KO mice to be more socially inhibited, and therefore display autistic-like symptoms. The statistical significance of less time spent in the center chamber was not

unexpected, as mice had previous exposure to the empty chamber and were expected to explore the chambers with a novel object or stranger mouse present. Males were also significantly less socially inhibited than females. This was seen in the first trial, as male mice spent a significantly greater amount of time with the stranger mouse than female mice did. However, in the second trial, female mice spent more time than male mice with the new stranger, indicating an increased social novelty seeking and increased recognition of social cues.

While wild-type data for the rotarod task has yet to be analyzed, effects can still be seen within the 5-HTT KO mice. The early prenatal stress group had a statistically significant shorter latency to fall, indicative of decreased locomotor coordination. The late prenatal stress group had a trend of a shorter latency to fall. Both results suggest that the presence of prenatal stress in the development of 5-HTT KO mice results in an impairment of cerebellar development, possibly similar to the cerebellar deficits seen in individuals with ASD.

Conclusions regarding the validity of this paradigm as an animal model of autism cannot be made until behavioral data of wild-type mice in early and late stress conditions are collected and analyzed. However, it appears that the suggested stress by 5-HTT KO genotype paradigm is an incomplete animal model of the symptoms of autism. Although the current findings do not allow for a conclusion that the model captures all aspects of the symptomology of autism, there were significant and interesting behavioral effects. Therefore, further investigations with 5-HTT KO mice and the effects of prenatal stress offer relevant and important future directions for research.

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